REMARKS

Following entry of the foregoing amendments, claims 108 to 119 will be pending in this patent application. Claims 108, 110, and 111 have been amended, herein. No claims have been canceled, and no new claims have been added. The claims have been amended to more clearly define the claimed invention and to correct typographical errors. No new matter has been added.

Applicants respectfully request reconsideration of the rejections of record in view of the foregoing amendments and the following remarks.

Claim Objections

Claims 110 and 111 have been objected to for containing an inadvertent misspelling of the word "internucleoside." These claims have been amended to correct the typographical error, obviating the objection. Applicants accordingly, respectfully request withdrawal thereof.

Alleged Anticipation

Claims 108 to 119 were rejected under 35 U.S.C. § 102(e) as allegedly anticipated by published U.S. patent application number US 2005/0142535 ("the Damha application"). Applicants respectfully request reconsideration and withdrawal of the rejection because the Damha application fails to describe or suggest every limitation of the claims.

Claim 108 recites methods of eliciting cleavage of a target RNA in a cell comprising contacting the cell with an oligomeric compound comprising a single-stranded oligonucleotide consisting of 12 to 30 linked nucleosides. The single-stranded oligonucleotide has a nucleobase sequence fully complementary to the nucleobase sequence of the target RNA, each nucleoside of the single-stranded oligonucleotide comprises a 2'-fluoro modification in the ribo configuration,[1] and at least one internucleoside linkage of the single-stranded oligonucleotide is a phosphorothioate linkage. Claim 119 depends from claim 108.

[1] Claim 108 has been amended herein to recite that each nucleoside of the single-stranded oligonucleotide comprises a 2'-fluoro modification *in the ribo configuration*. Support for the amendment is found throughout the specification as originally filed, including, for example, paragraph 78.

The Damha application fails to describe or suggest every element of the claimed methods. The rejected claims recite methods using a single-stranded oligonucleotides wherein each nucleoside comprises a 2'-fluoro modification in the ribo configuration. The Damha application, on the other hand, describes oligonucleotides that have alternating segments of sugar-modified nucleosides and unmodified 2'-deoxynucleosides.[2] Further, the Damha application reports results of experiments in which the ability of various antisense oligonucleotides to elicit RNase H[3] degradation of target RNA was evaluated.[4] The antisense oligonucleotides tested in the experiments included oligonucleotides having alternating segments of various lengths of 2'-deoxyribothymidine nucleosides (DNA) and 2'-deoxy-2'-fluoro-D-arabinothymidine nucleosides (FANA) and also included oligonucleotides containing all 2'-deoxyribothymidine nucleosides or all 2'-deoxy-2'-fluoro-D-arabinothymidine nucleosides.[5] None of those experiments included oligonucleotides in which every nucleoside comprises a 2'-fluoro modification in the ribo configuration. Indeed, since such a compound would not have been expected to support RNase H cleavage, there would have been no reason for such a compound to be included in this series of experiments designed to find RNase H dependent oligonucleotides. Thus, the Damha application fails to disclose the element of oligonucleotides wherein each nucleoside comprises a 2'-fluor modification in the ribo configuration. For at least that reason, the Damha application fails to anticipate the present claims.

Further, the rejected claims recite that "at least one internucleoside linkage of the single-stranded oligonucleotide is a phosphorothioate linkage." The compounds in the Damha application all contained only ***phosphodiester*** linkages.[6] For at least that reason, the Damha application fails to anticipate the present claims.

Moreover, the *in vitro* experiments described in the Damha application were not conducted in cells as recited in the rejected claims. Rather, the Damha application described mixing the oligonucleotides with complementary, labeled target oligoribonucleotides and then

[2] See, for example, paragraphs 14-42.
[3] *E. coli* RNase HI and human RNase HII.
[4] Examples 1-3.
[5] Table 1.
[6] *Id.*

heating and cooling the mixtures to allow formation of duplexes.[7] RNase H was then added to initiate a cleavage reaction and the reaction products were resolved by electrophoresis.[8] The Damha application reports that FANA/DNA chimeras induced target RNA cleavage by RNase H and cleavage efficiency increased as the size of the alternating segments increased.[9] Optimal activity was observed with an oligonucleotide that contained alternating trinucleotide segments of FANA and DNA, and the RNase H cleavage activity observed for this oligonucleotide was significantly better than the activity observed for the all-FANA oligonucleotide.[10] Significantly, these experiments were not conducted ***in a cell*** as recited in the present claims. For at least that reason, Damha fails to anticipate the present claims.

To anticipate, a reference must disclose every element of the invention as claimed. The rejected claims recite oligonucleotides wherein each nucleoside comprises a 2'-fluoro modification in the ribo configuration. The Damha application fails to disclose such oligonucleotides wherein each nucleoside comprises a 2'-fluoro modification in the ribo configuration. The rejected claims recite oligonucleotides comprising at least one phosphorothioate internucleoside linkage. The Damha application fails to discloses such oligonucleotides comprising at least one phosphorothioate internucleoside linkage. The rejected claims recite methods of eliciting cleavage of a target RNA in a cell. The Damha application fails to disclose such methods of cleavage in a cell. For at least those reasons, the Damha application thus fails to describe or suggest the claimed methods. Accordingly, applicants respectfully request withdrawal of the rejection for alleged anticipation based on the application.

Alleged Obviousness

Claims 108 to 111 have been rejected under 35 U.S.C. § 103(a) as allegedly rendered obvious by U.S. patent number 6,133,246 ("the McKay patent"), Lima, *et al.*, *Biochem.*, 1997, 36, 390-398 ("the Lima article"), Elbashir, *et al.*, *EMBO J.*, 2001, 20, 6877-6888 ("the Elbashir

[7] Example 1, paragraph 144.
[8] *Id.*
[9] Example 3 and Figure 2.
[10] *Id.*

article"), published U.S. patent application number 2007/0032446 ("the Cook application"), and the Damha application. Applicants respectfully request reconsideration and withdrawal of the rejection because the claimed methods would not have been obvious to those of ordinary skill in the art at the time of the invention.

To establish *prima facie* obviousness, the Patent Office must demonstrate that the cited prior art reference or combination of references teaches or suggests all the limitations of the claims.[11] The Patent Office must also identify "a reason that would have prompted a person of ordinary skill in the relevant field to combine the elements in the way the claimed new invention does."[12] In other words, the Office must identify "an apparent reason to combine the known elements *in the fashion claimed by the patent at issue*. To facilitate review, this analysis should be made explicit."[13]

As discussed above, the claims recite methods of eliciting cleavage of a target RNA in a cell that comprise contacting the cell with an oligomeric compound comprising a single-stranded oligonucleotide consisting of 12 to 30 linked nucleosides. The single-stranded oligonucleotide has a nucleobase sequence fully complementary to the nucleobase sequence of the target RNA, each nucleoside of the single-stranded oligonucleotide comprises a 2'-fluoro modification in the ribo configuration, and at least one internucleoside linkage of the single-stranded oligonucleotide is a phosphorothioate linkage.

Such methods would not have been obvious to those of ordinary skill in the art at the time of applicants' invention. The McKay patent, the Lima article, the Cook application, and the Damha application all describe compounds and methods for RNase H dependent antisense. It was widely known at the time of the invention that a minimum of five consecutive DNA-like nucleosides are needed in an antisense oligonucleotide to elicit target cleavage by human RNase H1, and a minimum of four consecutive DNA-like nucleosides are needed to elicit target

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[11] *In re Royka*, 490 F.2d 981, 180 U.S.P.Q. 580 (C.C.P.A. 1974); *In re Wilson*, 424 F.2d 1382, 1385, 165 U.S.P.Q. 494, 496 (C.C.P.A. 1970).
[12] *KSR Int'l Co. v. Teleflex*, 127 S.Ct. 1727, 1741.
[13] *KSR Int'l. Co. v. Teleflex Inc.*, 127 S. Ct. 1727, 1741 (emphasis added)(citing *In re Kahn*, 441, F.3d 977, 988 (Fed. Cir. 2006).

cleavage by *E. coli* RNase H1.[14] One skilled in the art designing an RNase H dependent antisense compound would have had no reason to try an oligonucleotide wherein each nucleoside comprises a 2'- fluoro modification, as such compounds lack the four or five consecutive DNA-like nucleoside segment necessary for RNase H cleavage and therefore would not have been expected to support RNase H cleavage.

The Elbashir article describes double-stranded siRNA duplexes. Since the present claims recite methods using single-stranded compounds, one of skill in the art would not look to Elabashir for guidance. Moreover, the Elbashir article shows that complete substitution of one or both strands of an siRNA duplex with 2'-modified nucleosides abolished the RNAi activity of the duplexes. Thus, one of ordinary skill in the art would not have been motivated to use fully 2'modified oligonucleotides as part of an siRNA duplex when attempting to elicit RNAi activity, much less as a single-stranded compound as claimed.

Those of ordinary skill in the art, when seeking to develop methods for eliciting cleavage of target RNA in cells, would have had no reason to make and use fully 2' modified single-stranded oligonucleotides as recited in the claimed methods. The cited art teaches away from such oligonucleotides for eliciting either RNase H cleavage or as part of a duplex to elicit siRNA activity. The claimed methods thus would not have been obvious to those of ordinary skill in the art at the time of the invention.

The Office contends, however, that the claimed methods are obvious because those of ordinary skill in the art would have expected that incorporation of 2' modifications into oligonucleotides would provide higher affinity for a target gene, enhanced stability, and/or enhanced cleavage of target genes.[15] As discussed above, however, one would not have expected such compounds to have been suitable for RNase H applications (since they lack a DNA gap) or for siRNA applications (because single-stranded and fully 2'-modified oligonucleotides were known to have little or no activity). Accordingly, in contrast to the Office's assertions, those of ordinary skill in the art would not have expected fully 2'-fluoro

[14] Wu, H., *et al.*, *J. Biol. Chem.*, 1999, 274, 28270-28272, attached as Appendix A.
[15] Office action dated February 6, 2008, page 7.

modified oligonucleotides to have been viable candidates for eliciting cleavage of a target RNA in a cell as presently claimed. The claimed methods thus would not have been obvious at that time, and applicants accordingly, respectfully, request withdrawal of the rejection.

Conclusion

Applicants believe that the foregoing constitutes a complete and full response to the official action of record. Accordingly, an early and favorable action is respectfully requested.

Respectfully submitted,

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/Jane E. Inglese/
Jane E. Inglese, Ph.D.
Registration No. 48,444

Woodcock Washburn LLP
Cira Centre
2929 Arch Street, 12th Floor
Philadelphia, PA 19104-2891
Telephone: (215) 568-3100
Facsimile: (215) 568-3439

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Properties of Cloned and Expressed Human RNase H1*

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Hongjiang Wu, Walt F. Lima, and Stanley T. Crooke‡

From Isis Pharmaceuticals, Inc., Carlsbad, California 92082

We have characterized cloned His-tag human RNase H1. The activity of the enzyme exhibited a bell-shaped response to divalent cations and pH. The optimum conditions for catalysis consisted of 1 mM Mg^{2+} and pH 7–8. In the presence of Mg^{2+}, Mn^{2+} was inhibitory. Human RNase H1 shares many enzymatic properties with *Escherichia coli* RNase H1. The human enzyme cleaves RNA in a DNA-RNA duplex resulting in products with 5′-phosphate and 3′-hydroxy termini, can cleave overhanging single strand RNA adjacent to a DNA-RNA duplex, and is unable to cleave substrates in which either the RNA or DNA strand has 2′ modifications at the cleavage site. Human RNase H1 binds selectively to "A-form"-type duplexes with approximately 10–20-fold greater affinity than that observed for *E. coli* RNase H1. The human enzyme displays a greater initial rate of cleavage of a heteroduplex-containing RNA-phosphorothioate DNA than an RNA-DNA duplex. Unlike the *E. coli* enzyme, human RNase H1 displays a strong positional preference for cleavage, *i.e.* it cleaves between 8 and 12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex. Within the preferred cleavage site, the enzyme displays modest sequence preference with GU being a preferred dinucleotide. The enzyme is inhibited by single-strand phosphorothioate oligonucleotides and displays no evidence of processivity. The minimum RNA-DNA duplex length that supports cleavage is 6 base pairs, and the minimum RNA-DNA "gap size" that supports cleavage is 5 base pairs.

RNase H1 hydrolyzes RNA in RNA-DNA duplexes (1). Proteins with RNase H activity have been isolated from numerous organisms ranging from viruses to mammalian cells and tissues (2–7). Although RNase H isotypes vary substantially in molecular weight and associated functions, the nuclease properties of the enzymes are similar. All RNase H enzymes, for example, function as endonucleases, specifically cleave RNA in RNA-DNA duplexes, require divalent cations, and generate products with 5′-phosphate and 3′-hydroxyl termini (7).

In prokaryotes, three classes of RNase H enzymes, RNase H1, H2, and H3, have been identified. RNase H2 and H3 share significant sequence homology, whereas RNase H3 and RNase H1 share similar divalent cation preference and cleavage properties. Of the three classes, RNase H2 appears to be the most ubiquitous (8). To date no organism has been shown to express active forms of all three classes of RNase H. The best characterized of the prokaryotic enzymes is *Escherichia coli* RNase H1 (9–13). This enzyme is believed to be involved in DNA replication (14). The key amino acids involved in metal binding, substrate binding, and catalysis have been identified and are highly conserved in the RNase H family (12, 15–17). Furthermore, the enzyme-substrate interaction has been elucidated based on both the three-dimensional structure of the enzyme as well as chemical and structural modification of the heteroduplex substrate (10, 13, 18–21).

RNase H has also been shown to be involved in viral replication. RNase H domains have been identified in viral reverse transcriptases, and these typically share homology with *E. coli* RNase H1 (15). The RNase H portion of the enzyme has been shown to cleave the viral RNA strand producing RNA primers for second strand DNA synthesis, thereby converting the viral RNA into double strand DNA (22).

Two classes of RNase H enzymes have been identified in mammalian cells (2–6). They were reported to differ with respect to co-factor requirements and activity. For example, RNase H type 1 has been shown to be activated by both Mg^{2+} and Mn^{2+} and was active in the presence of sulfhydryl reagents, whereas RNase H type 2 was shown to be activated by only Mg^{2+} and inhibited by Mn^{2+} and sulfhydryl reagents (6). Although the biological roles of the mammalian enzymes are not fully understood, it has been suggested that mammalian RNase H type 1 may be involved in replication and that the type 2 enzyme may be involved in transcription (25, 26).

Recently both human RNase H genes have been cloned and expressed (16, 17, 27). In a previous study we have reported the cloning and expression of a His-tag-labeled RNase H from human cells (16). The human enzyme was homologous to *E. coli* RNase H1. However, its biochemical properties were similar to those reported for the partially purified RNase H type 2. Because it was the first human enzyme to be cloned, it is referred to as human RNase H1. Additionally, a second human RNase H has been cloned (27)[1] but not yet been expressed in an active form. It was shown to be homologous to *E. coli* RNase H2 (28). It is referred to as human RNase H2.

In this communication we provide the first detailed characterization of the enzymological properties of human RNase H1 and compare its properties to those of the homologous protein *E. coli* RNase H1. These studies provide a basis to begin to develop a better understanding of the biological and pharmacological roles of the human RNase H family and to design antisense drugs that interact more effectively with the enzyme.

EXPERIMENTAL PROCEDURES

Materials—T4 polynucleotide kinase was purchased from Promega (Madison, WI). [γ-^{32}P]ATP and [^{32}P]cytidine bisphosphate were purchased from ICN (Irvine, CA). RNase inhibitor was from 5 Prime → 3 Prime, Inc. (Boulder, CO). Calf intestine alkaline phosphatase (CIP)[2] and T4 RNA ligase were purchased from Roche Molecular Biochemicals). Some oligodeoxynucleotides were purchased from Retrogen Inc. (San Diego, CA). The oligodeoxynucleotides were greater than 90% full-length material as determined by capillary gel electrophoresis anal-

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‡ To whom correspondence should be addressed: Isis Pharmaceuticals, Inc., 2292 Faraday Ave., Carlsbad, CA 92082. Tel.: 760-603-2301; Fax: 760-931-0265; E-mail: scrooke@isisph.com.

[1] H. Wu, unpublished data.

[2] The abbreviation used is: CIP, calf intestine alkaline phosphatase.

ysis. Human RNase H1 with a His-tag was expressed and purified from a bacterial expression system as described previously (16).

Oligonucleotide Synthesis—Synthesis of 2′-methoxy, 2′-fluoro, 2′-propoxy, and deoxy chimeric oligonucleotides was performed using an Applied Biosystems 380B automated DNA synthesizer as described previously (29, 30). Purification of oligonucleotides was also as described previously (29, 30). Purified oligonucleotides were greater than 90% full-length material as determined by capillary gel electrophoretic analysis.

^{32}P-labeling of RNA Transcripts and Oligoribonucleotides—RNA transcripts and oligoribonucleotides were 5′-end-labeled with ^{32}P using [γ-^{32}P]ATP and T4 polynucleotide kinase (31). Oligoribonucleotides were 3′-end-labeled using [^{32}P]cytidine bisphosphate and T4 RNA ligase. Labeled transcripts and oligonucleotides were purified by electrophoresis on 12% denaturing polyacrylamide gel. The specific activity of the 5′- and 3′-labeled RNAs were, respectively, approximately 6000 and 2000 cpm/fmol.

RNase H Assay Conditions—Hybridization reactions were performed in a variety of reaction buffers (20 mM Tris or NaH_2PO_4 buffer (pH 5.0–10.0), 0–10 mM $MgCl_2$, 0–5 mM $MnCl_2$, 20–120 mM KCl, 0–100 mM NaCl, 0–5 mM *N*-ethylmaleimide, 5% glycerol) containing 100 nM antisense oligonucleotide, 50 nM sense oligoribonucleotide, and 50,000 cpm (per 10-μl reaction volume) ^{32}P-labeled sense oligoribonucleotide. Reactions were heated at 90 °C for 2 min, then cooled, and RNase inhibitor, bovine serum albumin, and 2-mercaptoethanal (final concentration: 1 unit/100 μl, 10 ng/100 μl, and 5 mM, respectively) were added. Samples were equilibrated at 37 °C for at least 4 h and then incubated with human RNase H1. Samples were analyzed using the trichloroacetic acid assay as described previously and polyacrylamide gel electrophoresis (18, 21).

Determination of Initial Rates and Analysis of RNase H Cleavage Sites—Various substrates at different concentrations (10–500 nM RNA, 20–000 nM antisense oligonucleotide) were prepared as described above in the reaction buffer (20 mM Tris-HCl (pH 7.5), 1 mM $MgCl_2$, 20 mM KCl, 5% glycerol, 1 unit/100-μl RNase inhibitor, 10 ng/100-μl bovine serum albumin and 5 mM 2-mercaptoethanol). Substrates were incubated with human RNase H1 or *E. coli* RNase H1 and then quenched at specific times. Samples were analyzed by the trichloroacetic acid assay. The amount of substrate hydrolyzed was measured, and the initial rate and Michaelis-Menten parameters (K_m, V_{max}) were calculated (32). Substrate concentrations for trichloroacetic acid assays were the concentrations (nM) of intact duplex in an incubation. The trichloroacetic acid assay compares the amount of 5′ ^{32}P-labeled oligonucleotide that precipitates, thus directly measuring the fraction of duplex that remains intact, and by subtraction, the fraction cleaved to be trichloroacetic acid-soluble. Control studies showed that trichloroacetic acid precipitation was quantitative for single strand oligonucleotides ≥12 nucleotides in length. As the substrates were 5′-labeled, most cleavage products were trichloroacetic acid-soluble. For longer products, the trichloroacetic acid assay may underestimate cleavage; however, polyacrylamide gel electrophoretic analysis confirmed the cleavage rates observed in the trichloroacetic acid assays (data not shown). Consequently, the errors introduced into the trichloroacetic acid assay results by variations in precipitation of oligonucleotides of different lengths must be small. RNase H generated cleavage products were analyzed by a denaturing polyacrylamide gel. A base hydrolysis ladder was prepared by incubation of 5′-end-labeled RNA at 90 °C for 5 min in 100 mM $NaCO_3$ (pH 9.0). The positions of the cleavage sites were determined with oligonucleotide size markers generated by RNases A and T1 (33). The gels were then analyzed and quantified using a Molecular Dynamics PhosphorImager (21).

Determination of Binding Affinity—Binding affinities were determined by competitive inhibition analyses. At various concentrations ($n > 5$) ranging from 10 to 100 nM, the substrates, *i.e.* oligodeoxynucleotide-oligoribonucleotide hybrids, were prepared as described above. The competing substrate analog was prepared in reaction buffer containing equimolar concentrations of the modified sense and antisense oligonucleotides. Following equilibration at 37 °C, the competing substrate analog was added to the wild type substrate reaction, and the mixture was incubated with human RNase H1 in the presence of excess competing substrate, as described above. The samples were analyzed by trichloroacetic acid assay and denaturing polyacrylamide gel analyses. These data were analyzed by both the Lineweaver-Burk and Augustinsson methods to determine if the inhibitors were competitive and to ascertain the inhibitory constants (K_i) for the competing substrates, also as described previously (21, 32, 34).

RESULTS

Properties of Purified Human RNase H1—The effects of various reaction conditions on the activity of human RNase H1 were evaluated (Fig. 1). The optimal pH for the enzyme in both Tris-HCl and phosphate buffers was 7.0–8.0. At pH values above pH 8.0, enzyme activity was reduced. However, this could be due to instability of the substrate or effects on the enzyme, or both. To evaluate the potential contribution of changes in ionic strength to the activities observed at different pH values, two buffers, NaH_2PO_4 and Tris-HCl, were studied at pH 7.0 and gave the same enzyme activity even though the ionic strengths differed. Enzyme activity was inhibited by increasing ionic strength (Fig. 1*B*) and *N*-ethylmaleimide (Fig. 1*C*). Enzyme activity increased as the temperature was raised from 25 to 42 °C (Fig. 1*D*). Mg^{2+} stimulated enzyme activity with an optimal concentration of 1 mM. At higher concentrations, Mg^{2+} was inhibitory (Fig. 1*E*). In the presence of 1 mM Mg^{2+}, Mn^{2+} was inhibitory at all concentrations tested (Fig. 1*F*). The purified enzyme was quite stable and easily handled. In fact, the enzyme could be boiled and rapidly or slowly cooled without significant loss of activity (Fig. 1*D*). The initial rates of cleavage were determined for four duplex substrates studied simultaneously. The initial rate of cleavage for a phosphodiester DNA-RNA duplex was 1050 ± 203 pmol liter^{-1}min^{-1} (Table I*A*). The initial rate of cleavage of a phosphorothioate oligodeoxynucleotide duplex was approximately 4-fold faster than that of the same duplex comprised of a phosphodiester antisense oligodeoxynucleotide (Table I*A*). The initial rates for 17-mer and 20-mer substrates of different sequences were equal (Table I*B*). However, when a 25-mer heteroduplex containing the 17-mer sequence in the center of the duplex was digested (RNA No. 3), the rate was 50% faster. Interestingly, the K_m of the enzyme for the 25-mer duplex was 40% lower than that for the 17-mer, whereas the V_{max} values for both duplexes were the same (see Table III), suggesting that with the increase in length, a larger number of cleavage sites are available, resulting in an increase in the number of productive binding interactions between the enzyme and substrate. As a result, a lower substrate concentration is required for the longer duplex to achieve a cleavage rate equal to that of the shorter duplex.

To better characterize the substrate specificity of human RNase H1, duplexes in which the antisense oligonucleotide was modified in the 2′ position were studied. As previously reported for *E. coli* RNase H1 (18–21), human RNase H1 was unable to cleave substrates with 2′ modifications at the cleavage site of the antisense DNA strand or the sense RNA strand (Table II). For example, the initial rate of cleavage of a duplex containing a phosphorothioate oligodeoxynucleotide and its complement was 3400 pmol liter^{-1}min^{-1}, whereas that of its 2′-propoxy-modified analog was undetectable (Table II). A duplex comprised of a fully modified 2′-methoxy antisense strand also failed to support any cleavage (Table II). The placement of 2′-methoxy modifications around a central region of oligodeoxynucleotides reduced the initial rate (Table II). The smaller the central oligodeoxynucleotide "gap," the lower the initial rate. The smallest "gap-mer" for which cleavage could be measured was a 5 deoxynucleotide gap. These data are highly consistent with observations we have previously reported for *E. coli* RNase H1, except that for the bacterial enzyme, the minimum gap size was 4 deoxynucleotides (18, 20, 21).

The K_m and V_{max} of human RNase H1 for three substrates are shown in Table III. The K_m valves for all three substrates were substantially lower than those of *E. coli* RNase H1 (Table III) (18, 19). As previously reported for *E. coli* RNase H1, the K_m for a phosphorothioate-containing duplex was lower than

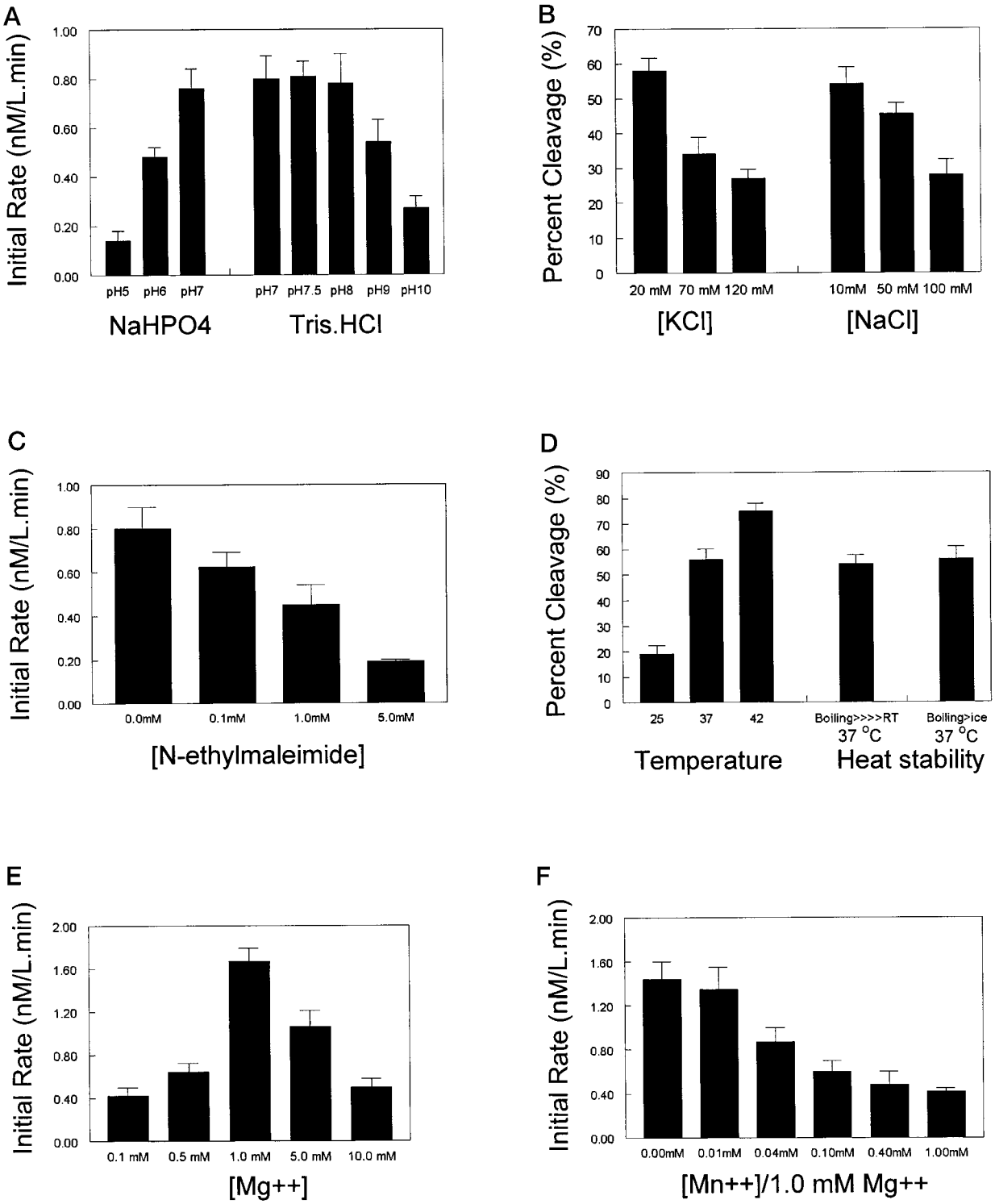

FIG. 1. **Effects of conditions on the human RNase H1 activity.** 5′-End-labeled RNA and antisense oligonucleotides were preannealed and digested with RNase H1 as described under "Experimental Procedures." The final substrate concentration was 20 nM for RNA and 40 nM for antisense oligonucleotide. The activity was measured as either initial rate or percent cleavage. *A*, pH dependence of RNase H activity. The substrate was annealed in phosphate or Tris buffer at different pH values and subjected to RNase H digestion in the presence of 10 mM Mg^{2+}. *B*, effect of ionic strength on RNase H activity. *C*, effect of the sulfhydryl-blocking agent, *N*-ethylmaleimide, on RNase H activity. The substrate was prepared in the same buffer as above without β-mercaptoethanol. *D*, temperature sensitivity and heat stability of the human RNase H1. Enzyme digestion was carried out under different temperatures. Alternatively, the enzyme was boiled for 5 min in buffer containing 50 mM Tris (pH 7.5), 50 mM NaCl, 1 mM EDTA, 20 mM dithiothreitol, and 50% glycerol, then either slowly cooled down to room temperature (*RT*) or rapidly moved into ice bath. *E*, effect of Mg^{2+} on RNase H activity. The substrate was prepared in the same buffer as above with a different concentration of Mg^{2+} and subject to RNase H digestion. *F*, effect of Mn^{2+} on RNase H1 activity. The substrate was digested in the buffer containing 1 mM Mg^{2+} and different concentrations of Mn^{2+}.

that of a phosphodiester duplex. The V_{max} of the human enzyme was 30-fold lower than that of the *E. coli* enzyme. The V_{max} for the phosphorothioate-containing substrate was less than the phosphodiester duplex. This is probably due to inhibition of the enzyme at higher concentrations by excess phosphorothioate single strand oligonucleotide (see below), as the

TABLE I

Effects of phosphorothioate substitution and substrate length on digestion by human RNase H1

Oligoribonucleotides were preannealed with the complementary antisense oligodeoxynucleotide at 10 and 20 nM and subjected to digestion by human RNase H1. The 17-mer (RNA No. 1) and 25-mer (RNA No. 3) RNA sequences are derived from Ha-Ras oncogen (51), and the 25-mer RNA contains the 17-mer sequence. The 20-mer (RNA No. 2) sequence is derived from human hepatitis C virus core protein coding sequence (52). The initial rates were determined as described under "Experimental Procedures." A, comparison of the initial rates of cleavage of an RNA-phosphodiester (P=O) and an RNA-phosphorothioate (P=S) duplexes. B, comparison among duplexes of different sequences and lengths.

RNA No.	RNA	Antisense DNA	Initial rate
			pmol liter^{-1} min^{-1}
A			
1	GGGCGCCGUCGGUGUGG	17-mer P=O	1050 ± 203
1	GGGCGCCGUCGGUGUGG	17-mer P=S	4034 ± 266
B			
1	GGGCGCCGUCGGUGUGG	17-mer P=O	1050 ± 203
2	ACUCCACCAUAGUACACUCC	20-mer P=O	1015 ± 264
3	UGGUGGGCGCCGUCGGUGUGGGCAA	25-mer P=O	1502 ± 182

TABLE II

Effects of 2′-substitution and deoxy-gap size on digestion rates by human RNase H1

Substrate duplexes were hybridized, and initial rates were determined as shown in Table I and described under "Experimental Procedures." The 17-mer RNA is the same used in Table I, and the 20-mer RNA (UGGUGGGCAAUGGGCGUGUU, RNA No. 4) was derived from the protein kinase C ζ (53) sequence. The 17-mer and 20-mer P=S oligonucleotides were full deoxyphosphorothioate-containing No. 2′-modifications. The 9, 7, 5, 4, and 3 deoxy gap oligonucleotides were 17-mer oligonucleotide with a central portion consisting of nine, seven, and five, and four deoxynucleotides flanked on both sides by 2′-methoexynucleotides (also see Fig. 2). Boldface sequences indicate the position of the 2′-methoxyl-modified residues. The italic sequences indicates the position of the 2′-propoxy-modified residues.

RNA No.	RNA	Antisense DNA	Initial Rate
			pmol liter^{-1} min^{-1}
1	17-mer	CCACACCGACGGCGCCC	4034 ± 266
	17-mer	**CCA**CACCGACGGC**GCCC**	1081 ± 168
	17-mer	**CCACA**CCGACGG**CGCCC**	605 ± 81
	17-mer	**CCACAC**CGACGG**CGCCC**	330 ± 56
	17-mer	**CCACAC**CGACG**GCGCCC**	0
	17-mer	**CCACAC**CGACG**GCGCCC**	0
	17-mer	**CCACACCGACGGCGCCC**	0
4*	20-mer	AACACGCCCATTGCCCACCA	3400 ± 384
	20-mer	*AACACGCCCATTGCCCACCA*	0

TABLE III

Kinetic constants for RNase H1 cleavage of RNA-DNA duplexes

The RNA-DNA duplexes in Table I were used to determine K_m and V_{max} of human and *E. coli* RNase H1 as described under "Experimental Procedures."

Substrates	Human RNase H		*E. coli* RNase H1	
	K_m		K_m	V_{max}
	nM	*nmol liter^{-1} min^{-1}*	nM	*nmol liter^{-1} min^{-1}*
25-mer Ras (RNA No. 3)-DNA (P=O)	35.4	1.907		
17-mer Ras (RNA No. 1)-DNA (P=O)	56.1	1.961	385	38.8
17-mer Ras (RNA No. 1)-DNA (P=S)	13.9	1.077		

initial rate of cleavage for a phosphorothioate-containing duplex was, in fact, greater than the phosphodiester (Table I)

Binding Affinity and Specificity—To evaluate the binding affinity of human RNase H1, a competitive cleavage assay in which increasing concentrations of noncleavable substrates were added was used (21). Using this approach, the K_i is formally equivalent to the K_d for the competing substrates. Of the noncleavable substrates studied, Lineweaver-Burk analyses demonstrated that all inhibitors shown in Table IV were competitive (data not shown). A duplex containing a phosphodiester oligodeoxynucleotide hybridized to a phosphodiester 2′-

TABLE IV

Binding constants and specificity of RNase H's

K_d values were determined as described under "Experimental Procedures." The K_d values for *E. coli* RNase H1 were derived from previously reported data (21). The competing substrates (competitive inhibitors) used in the binding study are divided into two categories: single strand (ss) oligonucleotides and oligonucleotide duplexes all with the 17-mer sequence as in Table 1 (RNA No. 1). The single strand oligonucleotides included ssRNA, ssDNA, ss fully modified 2′-methoxy phosphodiester oligonucleotide (ss 2′-methoxy), and ss full phosphorothioate deoxyoligonucleotide (ssDNA, P=S). The duplex substrates include DNA-DNA duplex, RNA-RNA duplex, DNA-fully modified 2′-fluoro or fully modified 2′-methoxy oligonucleotide (DNA-2′-fluoro or 2′-methoxy), RNA-2′-fluoro, or 2′-methoxy duplex. Dissociation constants are derived from ≥3 slopes of Lineweaver-Burk and/or Augustisson analysis. Estimated errors for the dissociation constants are ≤ 2-fold. Specificity is defined by dividing the K_d for a duplex by the K_d for an RNA-RNA duplex.

Inhibitors	Human RNase H1		*E.coli* RNase H1	
	K_d	Specificity	K_d	Specificity
	nM		nM	
DNA-2′-methoxy	458	5.8	3400	2.1
RNA-2′-methoxy	409	5.2	3100	1.9
RNA-RNA	79	1.0	1600	1.0
RNA-2′-fluoro	76	1.0		
DNA-2′-fluoro	99	1.3		
DNA-DNA	3608	45.7	176,000	110.0
ssRNA	1400	17.7		
ssDNA	1506	19.6	942,000	588.8
ss2′-methoxy	2304	29.2	118,000	73.8
ssDNA, P=S	36	0.5	14,000	8.8

methoxy oligonucleotide as the noncleavable substrate is considered most like DNA-RNA. Table IV shows the results of these studies and compares them to previously reported results for the *E. coli* enzyme performed under similar conditions (20, 21). Clearly, the affinity of the human enzyme for its DNA-RNA like substrate (DNA-2′-methoxy) was substantially greater than that of the *E. coli* enzyme, consistent with the differences observed in K_m (Table III).

E. coli RNase H1 displays approximately equal affinity for RNA-RNA, RNA-2′-methoxy, and DNA-2′-methoxy duplexes (Table IV). The human enzyme displays similar binding properties but is more able to discriminate between various duplexes. For example, the K_d for RNA-RNA was approximately 5-fold lower than the K_d for DNA-2′-methoxy. This is further demonstrated by the K_d for the RNA-2′-fluoro duplex. The K_d for the DNA-2′-fluoro duplex was slightly greater than for the RNA-2′-fluoro duplex and the RNA-RNA duplex but clearly lower than for other duplexes. Thus, both enzymes can be considered double strand RNA-binding proteins. However, human RNase H1 is somewhat less specific for duplexes as compared with single strand oligonucleotides than the *E. coli* enzyme. The enzyme bound to single strand RNA and DNA only

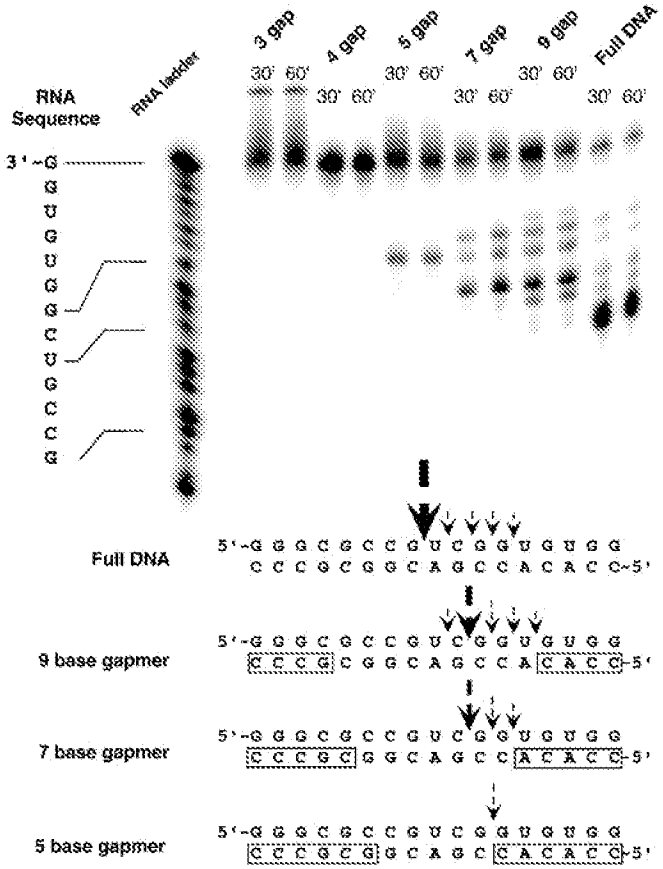

FIG. 2. **Denaturing polyacrylamide gel analysis of human RNase H1 cleavage of 17-mer RNA-DNA gap-mer duplex.** Antisense oligonucleotides were hybridized with 5′-end-labeled sense RNA as described under "Experimental Procedures," then digested with RNase H1 for 30 and 60 min at 37 °C. A base hydrolysis RNA ladder was prepared as described under "Experimental Procedures." The RNA ladder was sequenced with RNases T1, CL3, and A1 (data not shown). For each substrate, the RNA sequences (5′ → 3′) are shown above the DNA sequence. *Boxed sequences* indicate the position of the 2′-methoxy-modified residues. The *arrows* indicate the sites of the enzyme digestion, and the size of the *arrows* reflect the relative cleavage intensities.

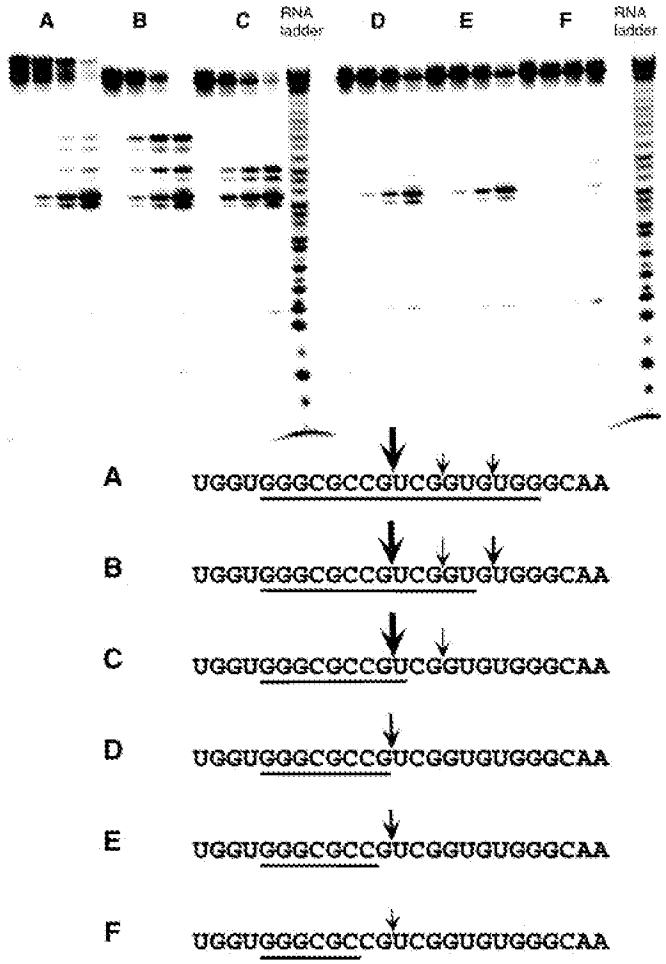

FIG. 3. **Analysis of human RNase H1 cleavage of a 25-mer Ras RNA hybridized with phosphodiester oligodeoxynucleotides of different lengths.** Antisense oligonucleotides with different lengths from 6- to 17-mer were hybridized with 5′-end-labeled 25-mer sense Ha-Ras RNA as described under "Experimental Procedures," then digested with RNase H1 at 37 °C for a time course of 0, 2, 5, and 10 min shown on the gel (left to right) for each substrate (*A* to *F*). A 25-mer RNA ladder was prepared and sequenced as described the legend for Fig. 2. For each substrate, the RNA sequences (5′ → 3′) are shown in the figure, and antisense DNA sequences were indicated by the *solid line* below the RNA sequence. The *arrows* indicate the sites of the enzyme digestion, and the size of *arrows* reflect the relative cleavage intensities.

20-fold less well than an RNA-RNA duplex, whereas the *E. coli* enzyme bound to single strand DNA nearly 600-fold less than to an RNA-RNA duplex (Table IV). The affinity of a single strand phosphorothioate oligodeoxynucleotide for both enzymes was significant relative to the affinity for the natural substrate and accounts for the inhibition of the enzymes by members of this class oligonucleotides. Remarkably, human RNase H1 displayed the highest affinity for a single strand phosphorothioate oligodeoxynucleotide. Thus, this noncleavable substrate is a very effective inhibitor of the enzyme, and excess phosphorothioate antisense drug in cells might be highly inhibitory.

Site and Sequence Preferences for Cleavage—Fig. 2 shows the cleavage pattern for RNA duplexed with its phosphorothioate oligodeoxynucleotide and the pattern for several gap-mers. In the parent duplex, RNA cleavage occurred at a single major site with minor cleavage noted at several sites 3′ to this major cleavage site that was 8 nucleotides from 5′ terminus of the RNA. Note that the preferred site occurred at a GU dinucleotide. Cleavage of several gap-mers occurred more slowly, and the major cleavage site was at a different position from that of the parent duplex. Furthermore, in contrast to the observations we have made for *E. coli* RNase H1 (18), the major cleavage site in gap-mers treated with human RNase H1 did not occur at the nucleotide apposed to the nucleotide adjacent to the first 2′-methoxy nucleotide in the wing hybridized to the 3′ portion of the RNA.

To further evaluate the site and sequence specificities of human RNase H1, cleavage of substrates shown in Figs. 3 and Fig. 4 was studied. In Fig. 3, the sequence of the RNA is displayed below the sequencing gels, and the length and position of the complementary phosphodiester oligodeoxynucleotide is indicated by the *solid line* below the RNA sequence. This figure demonstrates several important properties of the enzyme. First, the main cleavage site was consistently observed 8–9 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex irrespective of whether there were 5′ or 3′-RNA single strand overhangs. Second, the enzyme, like *E. coli* RNase H1 (20, 21), was capable of cleaving single strand regions of RNA adjacent to the 3′ terminus of an RNA-DNA duplex. Third, the minimum duplex length that supported any cleavage was approximately 6 nucleotides. RNase protection assays were used to confirm that under conditions of the assay, the shorter duplexes were fully hybridized, so the differences observed were not due to the failure to hybridize. To assure

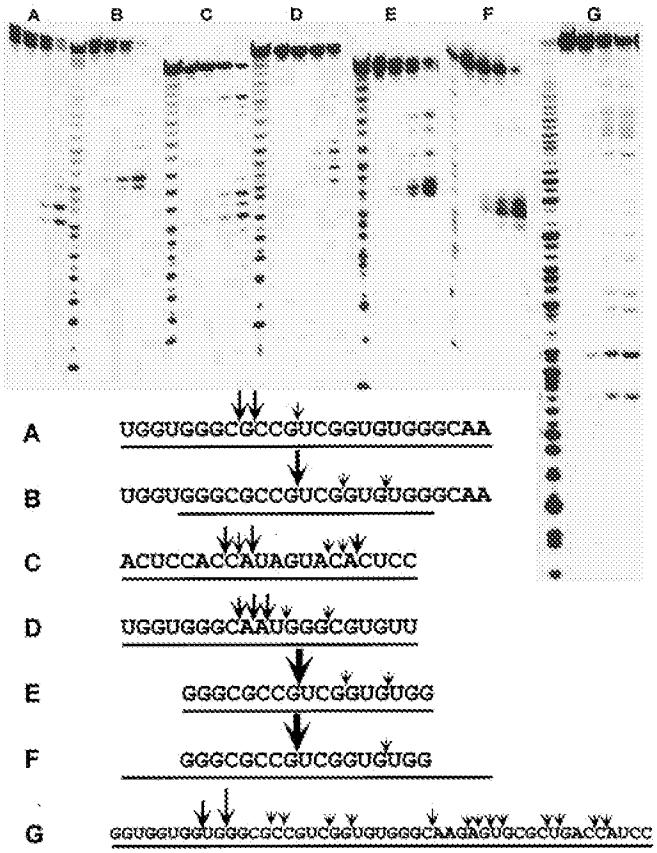

FIG. 4. **Analysis of human RNase H1 cleavage of RNA-DNA duplexes with different sequences, length, and 3′ or 5′ overhangs.** Antisense oligonucleotides of different sequences and lengths were hybridized with their complementary 5′-end-labeled RNA as described under "Experimental Procedures" and then digested with RNase H1 at 37 °C for 0, 2, 5, or 10 min as shown on the gel (left to right) for each substrate (*A* to *G*). Substrate *A* (25-mer), *B* (25-mer), *E* (17-mer), *F* (17-mer), *G* (47-mer) sequences are from the Harvey-RAS oncogene (51), substrate *C* (20-mer) is from hepatitis C virus (24), and substrate D (20-mer) is from protein kinase C ζ (23). The RNA ladder was prepared and sequenced as described in the legend for Fig. 2. For each substrate, the RNA sequences (5′ → 3′) are shown in the figure, and antisense DNA sequences were represented by the *solid line* below the RNA sequence. The *arrows* indicate the major sites and relative intensities of the enzyme digestion.

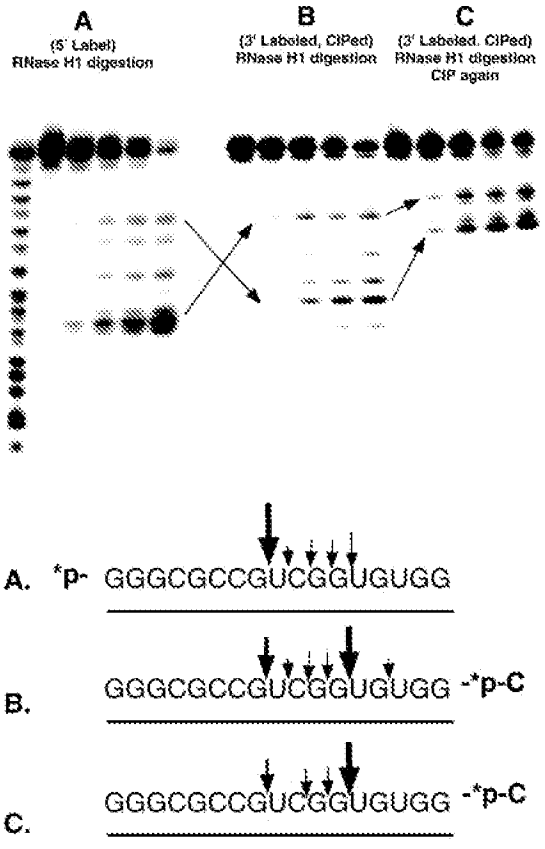

FIG. 5. **Product and processivity analysis of human RNase H1 cleavage on 17-mer Ras RNA-DNA duplexes.** RNA was either 5′-end-labeled (for *reaction A*) using [γ-^{32}P]ATP and T4 nucleotide kinase or 3′-end-labeled (for *reactions B* and *C*) using [^{32}P]cytidine bisphosphate and T4 RNA ligase as described under "Experimental Procedures." The 3′-end-labeled RNA was further dephosphorylated with calf intestine alkaline phosphatase (*CIPed*) (CIPed: dephosphorylated with CIP). Hybridization reactions were prepared as described in Fig. 1. The digestion with RNase H1 was performed at 37 °C for 0, 2, 5, 10, or 20 min as shown on the gel (left to right) for each substrate (*A* to *C*). Reactions with 3′-labeled substrate were divided into equal aliquots, with 1 aliquot subjected to further dephosphorylation with CIP. The **p-* indicates the position of the ^{32}P label. 5′- and 3′-end-labeled duplexes treated with human RNase H1 are shown in *panels A* and *B*, respectively. The 3′-end-labeled hybrid and degradation products treated with CIP after digestion with RNase H1 exhibited slower migration on the polyacrylamide gel due to the loss of the 5′-phosphate (*reaction C*) on the cleavage products. However, as the intact duplex had had its terminal phosphate removed by the previous CIP treatment (*panel C*), its migration was unchanged.

that the 6-nucleotide duplex was fully hybridized, the reactions were carried out at a 50:1 DNA-RNA ratio (data not shown). Fourth, the figure shows that for duplexes smaller than the nine base pairs, the smaller the duplex, the slower the cleavage rate. Fifth, the preferred cleavage site was located at a GU dinucleotide.

The site and sequence specificities are further explored in Fig. 4. That the enzyme displays little sequence preference is demonstrated by comparing the rates and sites of cleavage for duplexes A, C, and D. In all cases, the preferred site of cleavage was 8–12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex irrespective of the sequence. Comparison of the cleavage pattern for duplexes A and B shows that cleavage occurred at the 8–12 nucleotide position even when there were RNA overhangs also as shown in Fig. 3. Cleavage of duplex F demonstrated that the site of cleavage was retained even if there were 5′- and 3′-DNA overhangs. In a longer substrate, duplex G, the main site of cleavage was still 8–12 nucleotides from the terminus of the duplex. However, minor cleavage sites were observed throughout the RNA, suggesting that this substrate might support binding of more than one enzyme molecule/substrate, but that the preferred site was near the 5′-RNA-3′-DNA terminus. Finally, optimal cleavage seemed to occur when a GU dinucleotide was located 8–12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex.

To address both the mechanism of cleavage and processivity, the cleavage of 5′-labeled and 3′-labeled substrates was compared (Fig. 5). *Lane C* shows that CIP treatment before and after digestion with human RNase H1 resulted in a shift in the mobility of the digested fragments, suggesting that human RNase H1 generates cleavage products with 5′-phosphates. Thus, it is similar to *E. coli* RNase H1 in this regard (20). A second intriguing observation is that the addition of [^{32}P]cytidine to the 3′-end of the RNA caused a shift in the position of the preferred cleavage site (*A versus B* or *C*). The four cleavage sites in the center of the duplex observed with a 5′-phosphate-labeled RNA were observed in 3′-[^{32}P]cytidine-labeled substrates. However, the main cleavage site shifted from base pair 8 to base pair 12. Interestingly, the sequence at both sites was GU. Thus, it is conceivable that the enzyme selects a position 8–12 nucleotide from the 5′-RNA-3′-DNA terminus then

cleaves at a preferred dinucleotide such as GU. Third, this figure considered along with the cleavage patterns shown in Figs. 3 and 4 demonstrates that this enzyme displays minimal processivity in either the 5′ or 3′ direction. In no time-course experiment using any substrate have we observed a pattern that would be consistent with processivity. The possibility that the failure to observe processivity in Figs. 3 and 4 was due to processivity in the 3′ to 5′ direction is excluded by the results in Fig. 5. Again, this is significantly different from observations we have previously reported for *E. coli* RNase H1 (18).

DISCUSSION

General Properties of Human RNase H1 Activity—In this study, we have characterized the properties of human RNase H1. As the protein studied is a His-tag fusion and was denatured and refolded, it is possible that the activity of the enzyme in its native state might be greater than we have observed. However, basic properties reported in this paper are certainly likely to reflect the basic properties of the native enzyme. Numerous studies have shown that a His-tag does not interfere with protein folding and crystallization (35, 36), kinetic and catalytic properties (37, 38), or nucleic acid binding properties (39, 40),[3] since it is very small (few amino acids), and its pK is near neutral. As shown in this and our previous (16) studies, this His-tag fusion protein did behave like other RNase H proteins (6, 7). It cleaved specifically the RNA strand in RNA-DNA duplexes, resulted in cleavage products with 5′-phosphate termini (Fig. 5), and was affected by divalent cations (Fig. 1). Optimal conditions for human RNase H1 were similar but not identical to *E. coli* RNase H1. For the human enzyme, the Mg^{2+} optimum was 1 mM, and 5 mM Mg^{2+} was inhibitory. In the presence of Mg^{2+}, both enzymes were inhibited by Mn^{2+}. The human enzyme was inhibited by *N*-ethylmaleimide and was quite stable, easily handled, and did not form multimeric structures (Fig. 1). The ease of handling, denaturation, refolding, and stability in various conditions suggest that the human RNase H1 was active as a monomer and has a relatively stable preferred conformation.

Studies on the structure and enzymatic activities of a number of mutants of *E. coli* RNase H1 have recently led to a hypothesis to explain the effects of divalent cations termed an activation/attenuation model (41). The effects of divalent cations on human RNase H1 are complex and are consistent with the suggested activation/attenuation model. The amino acids proposed to be involved in both cation binding sites are conserved in human RNase H1 (16).

Positional and Sequence Preferences and Processivity—The site and sequence specificity of human RNase H1 differ substantially from *E. coli* RNase H1. Although neither enzyme displays significant sequence specificity (Ref. 18 and Figs. 2–5), the human enzyme displays remarkable site specificity. Figs. 2–4 show that human RNase H1 preferentially cleaved 8–12 nucleotides 3′ from the 5′-RNA-3′-DNA terminus of a DNA-RNA duplex irrespective of whether there were 5′ or 3′-RNA or DNA overhangs. The process by which a position is selected and then within that position on the duplex a particular dinucleotide is cleaved preferentially must be relatively complex and influenced by sequence. Clearly, the dinucleotide, GU, is a preferred sequence. In Fig. 3, for example, all the duplexes contained a GU sequence near the optimal position for the enzyme, and in all cases, the preferential cleavage site was GU. Additionally, in duplexes A and B a second GU was also cleaved, albeit at a very slow rate. The third site in duplexes A and B cleaved was a GG dinucleotide 7 base pairs from the 3′-RNA-5′-DNA terminus. Thus, the data suggest that the enzyme displays strong positional preference and, within the appropriate site, slight preference for GU dinucleotides.

The strong positional preference exhibited by human RNase H1 suggests that the enzyme fixes its position on the duplex via the 5′-RNA-3′-DNA terminus. Interestingly, the *in vitro* cleavage pattern observed for the enzyme is compatible with its proposed *in vivo* role, namely, the removal of RNA primers during DNA replication of the lagging strand. The average length of the RNA primer ranges from 7 to 14 nucleotides (42). Consequently, synthesis of the lagging strand results in chimeric sequences consisting of 7–14 ribonucleotides at the 5′ terminus with contiguous stretches of DNA extending in the 3′ direction. The positional preference observed for human RNase H1 (*i.e.* 8–12 residues from the 5′ terminus of the RNA) would suggest that cleavage of the chimeric lagging strand by RNase H1 would occur at or near the RNA-DNA junction. The removal of residual ribonucleotides following RNase H digestion has been shown to be performed by the endonuclease FEN1 (43).

Fig. 4 provides additional insight into the positional and sequence preferences of the enzyme. When there was a GU dinucleotide present in the correct position in the duplex, it was cleaved preferentially. When a GU dinucleotide was absent, AU was cleaved as well as other dinucleotides. For duplex G, both a GU and a GG dinucleotide were present within the preferred site, and in this case the GG dinucleotide was cleaved slightly more extensively than the GU dinucleotide. Clearly, additional duplexes of different sequences must be studied before definitive conclusions concerning the roles of various sequences within the preferred cleavage sites can be drawn.

In Fig. 5, the 3′ terminus of the RNA was labeled with [^{32}P]cytidine. In this case the same four nucleotides were cleaved as when the RNA was 5′-labeled (Fig. 5, *panels B* and *C*). However, the GU closer to the 3′ terminus of the RNA was cleaved at least as rapidly as the 5′-GU. Interestingly in studies on the partially purified enzyme, differences in the cleavage pattern were also observed when 5′-labeled substrates were compared with 3′-labeled substrates (6). At present, we have no explanation for this observation, but one possibility is that the presence of a 3′-phosphate on an oligonucleotide substrate affects the scanning mechanism the enzyme uses to select preferred positions for cleavage.

In a duplex comprised of RNA annealed to a chimeric oligonucleotide with an oligodeoxynucleotide center flanked by 2′-modified nucleotide wings, the cleavage by human RNase H1 was directed to the DNA-RNA portion of the duplex, as was observed for *E. coli* RNase H1 (18, 20). However, within this region, the preferred sites of cleavage for the human enzyme differed from *E. coli* RNase H1. *E. coli* RNase H1 preferentially cleaved at the ribonucleotide apposed to first 2′-modified nucleotide in the wing of antisense oligonucleotide at the 3′-end of the RNA (18). In contrast, the human enzyme preferentially cleaved at sites more centered within the gap until the gap was reduced to 5 nucleotides. Furthermore, the minimum gap size for the human enzyme was 5 nucleotides, whereas that of *E. coli* RNase H1 was 4 nucleotides (18). These differences in behavior suggest differences in the structures of the enzymes and their interactions with substrate that will require additional study.

We have reported that although *E. coli* RNase H1 degrades the heteroduplex substrate in a predominantly distributive manner, the enzyme displays modest 5′-3′ processivity. In contrast, human RNase H1 evidences no 5′-3′ or 3′-5′ processivity, suggesting that the human enzyme hydrolyzes the substrate in an exclusively distributive manner. The lack of processivity observed with the human RNase H1 may be a function of the significantly tighter binding affinity (Table IV), thereby reduc-

[3] L. B. Blyn, personal communication.

ing the ability of the enzyme to move on the substrate. Alternatively, human RNase H1 appears to fix its position on the substrate with respect to the 5′-RNA-3′-DNA terminus, and this strong positional preference may preclude cleavage of the substrate in a processive manner (Fig. 5). Thus, despite the facts that the enzymes are both metal-dependent endonucleases that result in cleavage products with 5′-phosphates (Fig. 5) and both can cleave single strand 3′-RNA overhangs (Fig. 5 and Ref. 20), these enzymes display substantial differences.

E. coli RNase H1 has been suggested to exhibit "binding directionality" with respect to the RNA of the substrate such that the primary binding region of the enzyme is positioned several nucleotides 5′ to the catalytic center (13). This results in cleavage sites being restricted from the 5′-RNA-3′-DNA end of a duplex and cleavage sites occurring at the 3′-RNA-5′-DNA end of the duplex and in 3′ single strand overhangs. The human enzyme behaves entirely analogously. Thus, we conclude that human RNase H1 likely has the same binding directionality as the *E. coli* enzyme.

Substrate Binding—RNA-RNA duplexes have been shown to adopt an A-form conformation (44, 45). Many 2′ modifications shift the sugar conformation into a 3′-endo pucker characteristic of RNA (9, 46–48). Consequently, when hybridized to RNA, the resulting duplex is A form, and this is manifested in a more stable duplex. 2′-fluoro oligonucleotides display duplex-forming properties most like RNA, whereas 2′-methoxy oligonucleotides result in duplex intermediate information between DNA-RNA and RNA-RNA duplexes (20).

The results shown in Table IV demonstrate that like the *E. coli* enzyme, human RNase H1 is a double strand RNA-binding protein. Moreover, it displays some ability to discriminate between various A-form duplexes (Table IV). The observation that the K_d for an RNA-2′-F duplex is equal to that for an RNA-RNA duplex suggests that 2′-hydroxy group is not required for binding to the enzyme. Nevertheless, we cannot exclude the possibility that bulkier 2′ modifications, e.g. 2′-methoxy or 2′-propyl, might sterically inhibit the binding of the enzyme as well as alter the A-form quality of the duplex. The human enzyme displays substantially greater affinity for all oligonucleotides than the *E. coli* enzyme, and this is reflected in a lower K_m for cleavable substrates (Tables III and IV). In addition, the tighter binding affinity observed for human RNase H1 may be responsible for the 20-fold lower V_{max} when compared with the *E. coli* enzyme. In this case, assuming that the *E. coli* and human enzymes exhibit similar catalytic rates (K_{cat}), then an increase in the binding affinity would result in a lower turnover rate and ultimately a lower V_{max}.

The principal substrate binding site in *E. coli* RNase H1 is thought to be a cluster of lysines that are believed to bind to the phosphates of the substrates (13). The interaction of the binding surface of the enzyme and substrate is believed to occur within the minor groove. This region is highly conserved in the human enzyme (16). In addition, eukaryotic enzymes contain an extra N-terminal region of variable length containing an abundance of basic amino acids (16, 17). This region is homologous with a double strand RNA binding motif and indeed in the *Saccharomyces cerevisiae* RNase H has been shown to bind to double strand RNA (17, 49). The N-terminal extension in human RNase H1 is longer than that in the *S. cerevisiae* enzyme and appears to correspond to a more complete double strand RNA binding motif. Consequently, the enhanced binding of human RNase H1 to various nucleic acids may be due to the presence of this additional binding site.

Biological Roles and Implications for Antisense Drug Design—As discussed previously, the positional preferences of human RNase H1 argue that the proposal that it may be involved in DNA replication may be correct (42). However, the lack of processivity would suggest that the enzyme is suboptimally designed for this task, but considering the involvement of FEN1 in DNA replication, processive cleavage of the RNA by RNase H may be unnecessary. Clearly, more work is required before any conclusions can be drawn.

Although RNase H enzymes have been suggested to be involved in the effects of DNA-like antisense drug, to date no studies have directly demonstrated this nor determined which isotypes may be involved. We now have the tools to begin to answer these questions. If human RNase H1 is involved, our studies suggest that excess single strand phosphorothioate oligonucleotides in cells would be highly inhibitory, resulting in loss of effectiveness at higher concentrations. Furthermore, the binding preference human RNase H1 displays for A-form duplexes suggests that binding of the enzyme would be enhanced by appropriate 2′ modifications. However, cleavage rates are lower in chimeric duplexes, so the design of optimal 2′-modified gap-mers may be challenging.

Clearly, if the positional and sequence preferences observed for oligonucleotide substrates were for RNA species bound to DNA-like antisense drugs, the implications would be substantial. For example, the placement of DNA gaps centered around a GU dinucleotide would be of value. Furthermore, since the positional preference of the enzyme was evident even when there were 5′- and 3′-RNA overhangs, positioning DNA gaps 8–12 nucleotides from the 5′-RNA-3′-DNA terminus of the duplex and creating a GU within that area could be beneficial. Also, locating antisense drugs at the 5′-end of an RNA should be of value. However, it is clear that many DNA-like antisense drugs bind to RNA species at sites distal from the 5′ terminus of the RNA and still result in loss of RNA, presumably via RNase H-mediated cleavage (50). Thus, much more work is required before conclusions can be drawn and the information can be used to design better antisense drugs.

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REFERENCES

1. Stein, H., and Hausen, P. (1969) *Science* **166,** 393–395
2. Eder, P. S., and Walder, J. A. (1991) *J. Biol. Chem.* **266,** 6472–6479
3. Busen, W. (1980) *J. Biol. Chem.* **255,** 9434–9443
4. Masutani, C., Enomoto, T., Suzuki, M., Hanaoka, F., and Ui, M. (1990) *J. Biol. Chem.* **265,** 10210–10216
5. Kane, C. M. (1988) *Biochemistry* **27,** 3187–3196
6. Frank, P., Albert, S., Cazenave, C., and Toulme, J. J. (1994) *Nucleic Acids Res.* **22,** 5247–5254
7. Crouch, R. J., and Dirksen, M. L. (1982) *Cold Spring Harbor Monogr. Ser.* **14,** pp. 211–254
8. Ohtani, N., Haruki, M., Morikawa, M., Crouch, R. J., Itaya, M., and Kanaya, S. (1999) *Biochemistry* **38,** 605–618
9. Nakamura, H., Oda, Y., Iwai, S., Inoue, H., Ohtsuka, E., Kanaya, S., Kimura, S., Katsuda, C., Katayanagi, K., Morikawa, K., Miyashiro, H., and Ikehara, M. (1991) *Proc. Natl. Acad. Sci. U. S. A.* **88,** 11535–11539
10. Katayanagi, K., Miyagawa, M., Matsushima, M., Ishikawa, M., Kanaya, S., Ikehara, M., Matsuzaki, T., and Morikawa, K. (1990) *Nature* **347,** 306–309
11. Kanaya, S., Katsuda-Nakai, C., and Ikehara, M. (1991) *J. Biol. Chem.* **266,** 11621–11627
12. Kanaya, S., Kohara, A., Miura, Y., Sekiguchi, A., Iwai, S., Inoue, H., Ohtsuka, E., and Ikehara, M. (1990) *J. Biol. Chem.* **265,** 4615–4621
13. Kanaya, S. (1998) in *Ribonucleases H* (Crouch, R. J., ed) pp. 1–38, INSERM, Paris
14. Kogoma, T., and Foster, P. L. (1998) in *Ribonucleases H* (Crouch, R. J., ed) pp. 39–66, INSERM, Paris
15. Yang, W., and Steitz, T. A. (1995) *Structure* **3,** 131–134
16. Wu, H., Lima, W. F., and Crooke, S. T. (1998) *Antisense Nucleic Acid Drug Dev.* **8,** 53–61
17. Cerritelli, S. M., and Crouch, R. J. (1998) *Genomics* **53,** 307
18. Crooke, S. T., Lemonidis, K. M., Neilson, L., Griffey, R., Lesnik, E. A., and Monia, B. P. (1995) *Biochem. J.* **312,** 599–608
19. Lima, W. F., Mohan, V., and Crooke, S. T. (1997) *J. Biol. Chem.* **272,** 18191–18199
20. Lima, W. F., and Crooke, S. T. (1997) *J. Biol. Chem.* **272,** 27513–27516
21. Lima, W. F., and Crooke, S. T. (1997) *Biochemistry* **36,** 390–398
22. Hughes, S. H., Arnold, E., and Hostomsky, Z. (1998) in *Ribonucleases H*

(Crouch, R. J., ed) pp. 195–224, INSERM, Paris
23. Liao, D.-F., Monia, B., Dean, N., and Berk, B. C. (1997) *J. Biol. Chem.* **272,** 6146–6150
24. Hanecak, R., Brown-Driver, V., Fox, M. C., Azad, R. F., Furusako, S., Nozaki, C., Ford, C., Sasmor, H., and Anderson, K. P. (1996) *J. Virol.* **70,** 5203–5212
25. Busen, W., Peters, J. H., and Hausen, P. (1977) *Eur. J. Biochem.* **74,** 203–208
26. Turchi, J. J., Huang, L., Murante, R. S., Kim, Y., and Bambara, R. A. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 9803–9807
27. Frank, P., Braunshofer-Reiter, C., Wintersberger, U., Grimm, R., and Busen, W. (1998) *Proc. Natl. Acad. Sci. U. S. A.* **95,** 12872–12877
28. Itaya, M. (1990) *Proc. Natl. Acad Sci. U. S. A.* **87,** 8587–8591
29. Kawasaki, A. M., Casper, M. D., Freier, S. M., Lesnik, E. A., Zounes, M. C., Cummins, L. L., Gonzalez, C., and Cook, P. D. (1993) *J. Med. Chem.* **36,** 831–841
30. Guinosso, C. J., Hoke, G. D., Frier, S., Martin, J. F., Ecker, D. J., Mirabelli, C. K., Crooke, S. T., and Cook, P. D. (1991) *Nucleosides Nucleotides* **10,** 259–262
31. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (1995) *Current Protocols in Molecular Biology, Third Edition*, p. 3.10.3, John Wiley & Sons, Inc., New York
32. Copeland, R. A., ed (1996) *Enzymes: A Practical Introduction to Structure, Mechanism, and Data Analysis*, VCH Publishers, Inc., New York
33. Lima, W. F., Monia, B. P., Ecker, D. J., and Freier, S. M. (1992) *Biochemistry* **31,** 12055–12061
34. Piszkiewicz, D. (1977) *Kinetics of Chemical and Enzyme-catalyzed Reactions*, p. 80–145, Oxford University Press, New York
35. Flachmann, R., and Kuehlbrandt, W. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 14966–14971
36. Lindner, P., Guth, B., Wuelfing, C., Krebber, C., Steipe, B., Mueller, F., and Plueckthun, A. (1992) *Methods (San Diego)* **4,** 41–56
37. Sonnenburg, W. K., Seger, D., Kwak, K. S., Huang, J., Charbonneau, H., and Beavo, J. A. (1995) *J. Biol. Chem.* **270,** 30989–31000
38. Sarcevic, B., Erikson, E., and Maller, J. L. (1993) *J. Biol. Chem.* **268,** 25075–25083
39. Blyn, L. B., Swiderek, K. M., Richards, O., Stahl, D. C., Semler, B. L., and Ehrenfeld, E. (1996) *Proc. Natl. Acad. Sci. U. S. A.* **93,** 11115–11120
40. Hirata, Y., Kiuchi, K., Chen, H. C., Milbrandt, J., and Guroff, G. (1993) *J. Biol. Chem.* **268,** 24808–24812
41. Keck, J. L., Goedken, E. R., and Marqusee, S. (1998) *J. Biol. Chem.* **273,** 34128–34133
42. Bambara, R. A., Murante, R. S., and Henricksen, L. A. (1997) *J. Biol. Chem.* **272,** 4647–4650
43. Barnes, C. J., Wahl, A. F., Shen, B., Park, M. S., and Bambara, R. A. (1996) *J. Biol. Chem.* **271,** 29624–29631
44. Kogoma, T., Subia, N. L., and von Meyenburg, K. (1985) *Mol. Gen. Genet.* **200,** 103–109
45. Inoue, H., Hayase, Y., Iwai, S., and Ohtsuka, E. (1987) *FEBS Lett.* **215,** 327–330
46. Morvan, F., Sanghvi, Y. S., Perbost, M., Vasseur, J.-J., and Bellon, L. (1996) *J. Am. Chem. Soc.* **118,** 255–256
47. Itoh, H., Mukoyama, M., Pratt, R. E., and Dzau, V. J. (1992) *Biochem. Biophys. Res. Commun.* **188,** 1205–1213
48. Tomizawa, J. (1990) *J. Mol. Biol.* **212,** 683–694
49. Cerritelli, S. M., Fedoroff, O. Y., Reid, B. R., and Crouch, R. J. (1998) *Nucleic Acids Res.* **26,** 1834–1840
50. Crooke, S. T. (1998) *Antisense Nucleic Acid Drug Dev.* **8,** 115–122
51. Monia, B. P., Johnston, J. F., Sasmor, H., and Cummins, L. L. (1996) *J. Biol. Chem.* **271,** 14533–14540